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WO 9604311A1

INTERNATIONAL APPLICATION PUBLISHED U...

(51) International Patent Classification 6 :
C07K 16/18, G01N 33/53, 33/531, 33/532, 33/558, 33/60, C12N 5/12

A1

(11) International Publication Number: WO 96/04311

(43) International Publication Date: 15 February 1996 (15.02.96)

(21) International Application Number: PCT/US95/09843

(22) International Filing Date: 3 August 1995 (03.08.95)

(30) Priority Data:
08/285,414 3 August 1994 (03.08.94) US

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(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: MONOCLONAL ANTIBODY TO NITROTYROSINE, METHODS FOR DIAGNOSIS AND METHODS FOR TREATMENT OF DISEASE

(57) Abstract

Nitrotyrosine residues in proteins are shown by histological staining studies to be a marker for oxidative damage to tissues in animals and in humans. Nitrotyrosine at sites of inflammation or in tumors can be detected by monoclonal antibodies specific to nitrotyrosine. Such antinitrotyrosine antibodies can be used in standard immunological methods for qualitative or quantitative assays of nitrotyrosine to diagnose various pathological conditions. The antibodies can be conjugated to various diagnostic or therapeutic agents, and the conjugates used to direct the diagnostic or therapeutic agents to sites of oxidative damage in living animals or humans.

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MONOCLONAL ANTIBODY TO NITROTYROSINE, METHODS FOR DIAGNOSIS AND METHODS FOR TREATMENT OF DISEASE

Government Support

This work was supported in whole or in part by National Institutes of Health Grant NIH 5P01 HL48676. The Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

There is increasing awareness that nitric oxide (NO) contributes to the pathophysiology of chronic inflammatory diseases, with the inducible form of nitric oxide synthase (NOS) proposed to play a pro-inflammatory role. (Miller, M.J.S., and D.A. Clark, *Agents Actions* In Press, 1994). Under basal or physiological conditions, nitric oxide is formed by constitutively expressed isoforms. These forms of NOS are commonly referred to as neuronal and endothelial NOS because of the cell types initially found to express them (Nathan, C., *FASEB J.* 6:3051-3064 (1992)). Neuronal and endothelial NOS release small amounts of nitric oxide in response to increased intracellular calcium; released NO in turn elevates intracellular cGMP in the target cell, thereby inducing vascular or intestinal smooth muscle relaxation or modifying neurotransmission. Because the neuronal and endothelial forms of NOS possess similar characteristics, they are often collectively classed as constitutive or cNOS. Macrophage NOS is known to be inducible.

Biochemical evidence of iNOS activity has been observed in human inflammatory bowel disease (Boughton-Smith, N.K, *et*

al., Lancet 341:338-340 (1993)) and elevated formation of nitric oxide has been found in a variety of induced or spontaneous forms of gut inflammation in animals (Miller, M.J.S., *et al., J. Pharmacol. Exp. Therapy 264*:11-16 (1993)); Ribbons, K.A., *et al., Gastroenterology 106*:A760 (1994); Grisham, M.B., *Curr. Op. Gastroenterology 9*:524-533 (1993)) and humans (Middleton, S.J., *et al., Lancet 341*:465-466 (1993); Roediger, W.E.W., *et al., Digestion 35*:199-204 (1986)). These could be epiphenomena were it not for the finding that inhibition of NOS ameliorates gut inflammation (Miller, M.J.S., *et al., J. Pharmacol. Exp. Therapy 264*:11-16 (1993)). The expression of iNOS or its consequences have been observed in adjuvant arthritis (Ialenti, A., *et al., Br. J. Pharmacol. 110*:701-706 (1993); McCartney-Francis, N., *et al., J. Exp. Med. 178*:749-754 (1993)), immune models of glomerulonephritis (Pfeischifter, J., *et al., Nephron 64*:518-525 (1993)) and the peptido-polysaccharide model of granulomatous colitis (Grisham, M.G., and Zimmerman, T.E., *Gastroenterology 106*:A696 (1994)). Although iNOS gene expression has been confirmed in adjuvant arthritis (Ialenti, A., *et al., Br. J. Pharmacol. 110*:701-706 (1993)) the evidence of iNOS gene expression in chronic inflammatory bowel disease has been confined to biochemical measures of enzyme activity.

Inducible NOS causes the release of far greater amounts of nitric oxide than does cNOS, in keeping with its role in host defense where it serves as a cytostatic mediator against tumors or invading microooganisms (Nathan, C. *FASEB J. 6*:3051-3064 (1992)). While the ability of the gut to express iNOS may be a vital means of establishing a hostile chemical barrier to luminal bacteria, induction of this pathway is not without potential complications. As in other pathways of host defense, intestinal tissue may be subjected to injury when iNOS is expressed. Under these conditions, NO-dependent actions are based in free radical chemistry rather than due to the activation of guanylate cyclase as is the case with cNOS.

It is becoming increasingly clear that the potency of nitric oxide as a free radical is insufficient to explain NO-

mediated cytotoxicity. Other oxidants or free radicals derived from nitric oxide must be involved. While nitric oxide will lead to the formation of a host of reactive nitrogen intermediates, one of the most attractive as a potential mediator of cytotoxicity is peroxynitrite.

Peroxynitrite has a pK of 6.8 at 37 °C and, when protonated, decomposes to generate highly reactive agents: apparently hydroxyl radical (HO·) and nitrogen dioxide (NO_2). Moreover, ONOO- has a half-life of under 1 second at pH 7.4, allowing it to diffuse to critical cellular targets before decomposing. It has been shown that classical ·OH scavengers competitively inhibit oxidation initiated by ONOO- decomposition, while the iron chelator desferrioxamine is a potent scavenger because of its direct reaction with ONOO-, independent of its ability to bind iron.

Macrophages and neutrophils produce both O_2- and ·NO when activated (Marletta, M.A., *et al.*, *Biochemistry 27*:8706-8711 (1988); Moncada, S., *et al.*, *Biochem. Pharmacol. 38*:1709-1715 (1989)), suggesting that ONOO- may be an additional cytotoxic agent formed by these cell types. In endothelium and brain, Ca^{2+} is the intracellular messenger initiating the NADPH-dependent oxidation of arginine to produce ·NO (Moncada, S., *et al.*, *Biochem. Pharmacol. 38*:1709-1715 (1989)). Pathophysiological processes such as ischemia, excessive activation of the N-methyl-D-aspartic acid receptor in brain, and sepsis allow the influx of Ca^{2+} into cells, which may stimulate the simultaneous production of ·NO and O_2–. Injurious amounts of ONOO– could result, because every ten-fold increase in the concentration of ·NO and O_2– will increase $ONOO^-$ formation 100-fold.

Such a mechanism may be important for reperfusion injury to ischaemic tissue, as ischaemic endothelium will accumulate Ca^{2+} and the substrates (arginine and NADPH) needed for NO synthesis but will produce ·NO or O_2– only when oxygen is supplied by reperfusion. Peroxynitrite efficiently oxidizes the fluorescent probe dihydrorhodamine 123, and has strong oxidizing properties towards biological molecules, including

protein and non-protein sulfhydryls, deoxyribonucleic acid, and membrane phospholipids (Kooy, N.W., *et al., Free. Rad. Biol. Med. 16*:149-156 (1994); Radi, R., *et al., J. Biol. Chem. 266*:4244-4250 (1991); King, P.A., *et al., J. Am. Chem. Soc. 114*:5430-5432 (1992); Radi, R., *et al., Arch. Biochem. Biophys. 288*:481-487 (1991)). In addition, peroxynitrite also nitrates free or protein-associated tyrosines and other phenolics via the metal-catalyzed formation of the nitronium ion (Ischiropoulos, H., *et al., Arch. Biochem. Biophys. 298*:431-437 (1992)). Phorbol 12-myristate 13-acetate activated alveolar macrophages produce peroxynitrite as demonstrated by the nitration of phenolic rings (Ischiropolous, H, *et al., Arch. Biochem. Biophys. 298*:446-451 (1992)). Therefore, inflammatory mediator-enhanced cellular production of nitric oxide and superoxide may lead to formation of peroxynitrite *in vivo*, and peroxynitrite, as a potent oxidant and nitrating agent, may serve an important role in a number of pathological conditions, among them, acute lung injury. Lipid peroxidation in humans with acute lung injury implies the action of a potent oxidant (Lemonnier, Richard C. *et al., Crit. Care Med., 18*:4-9 (1990)). However, the exact nature of the toxic oxidant remains uncertain. Data from animal models of acute lung injury suggest a prominent role for a nitric-oxide dependent oxidant such as peroxynitrite (Mulligan, M.S. *et al., Proc. Natl. Acad. Sci. USA, 88*:6338-6342 (1991)).

Superoxide dismutase has been found to catalyze the nitration of tyrosine by peroxynitrite (Ischiropoulos, H., *et al., Arch. Biochem. Biophys. 298*:431-437 (1992)). Small amounts of nitrotyrosine and its natural metabolites have been found in the urine of healthy humans by a sensitive gas chromatographic-thermal energy analysis method (Ohshima, H. *et al. Fund. Chem. Tox 28*:647-652 (1990).

Tyrosine nitration may have a biologic effect on the structure and function of proteins. Nitration of surfactant protein A by peroxynitrite has been associated with decreased protein function (Haddad, I.Y., *et al., Am J. Physiol. 265* (Lung Cell Mol Physiol 9):L555-L564 (1993)). Similarly,

nitration of tyrosine inhibits cytochrome P450 (Janing, G.R., *et al., Biochem. Biophys. Acta 916*:512-523 (1987)) and inactivates the complement subcomponent C1q binding capacity of human IgG (McCall, M.N., *et al., Biochem. J. 257*:845-851 (1989)). Tyrosine nitration *in vitro* by tetranitromethane has been shown to block phosphorylation of tyrosines by EGF (Martin, B.L. *et al., J. Biol. Chem., 265*:7108-7111 (1990)). The disruption of tyrosine kinase phosphorylation by nitration may be a critical pathological event resulting from the simultaneous production of superoxide and nitric oxide. Addition of water across the oxygen-phosphorous bond in phosphotyrosine regenerates tyrosine. In contrast, the nitration of tyrosine involves breaking a carbon-nitrogen bond, which is energetically expensive. Thus, nitration is not readily reversible and most likely requires degradation of the whole protein rather than removal of the nitro-group.

SUMMARY OF INVENTION

Monoclonal antibodies which specifically recognize and bind to nitrotyrosine and the nitrotyrosine residues within proteins are disclosed. The hybridoma cell line for their production is also disclosed. Antibodies specific for nitrotyrosine which have been linked to various substances to make conjugates useful in diagnosis and therapy of disease are also a part of the invention. Antinitrotyrosine antibodies are useful in methods for detecting various pathological conditions, especially inflammation and tumors. They are also useful in methods for specifically delivering therapeutic agents to sites of oxidative damages to tissues.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a western blot examining cross-reactivity of the antinitrotyrosine monoclonal antibody with bovine serum albumin (lanes b to e), rat brain homogenate (lanes f to i) and rat heart homogenate (lanes j to m). For each type of sample, the first lane was the untreated protein followed sequentially by the proteins exposed to 10 mM xanthine oxidase plus 100 μM

pterine and 100 μM Fe^{3+}EDTA, 5 mM hypochlorous acid or 1 mM peroxynitrite.

Figure 2 is a contour plot of fluorescence intensity of lung sections from an ARDS patient (C.D.; see Table 2) incubated with non-immune IgG or the polyclonal antibody raised against nitrotyrosine, followed by secondary antibody. The x-axis represents the intensity value for the number of pixels shown on the y-axis.

Figure 3 is a bar graph representing the quantification of immunofluorescence intensity for lung sections from ARDS patients (n=5) and for controls (non-ARDS; n=5), incubated with NTAb, an equivalent amount of non-immune IgG, or NTAb in the presence of excess nitrotyrosine, followed by the secondary antibody.

Figure 4 is a bar graph representing the group mean quantification of immunofluorescence intensity for hyperoxic and control lung sections treated with NTAb, an equivalent amount of non-immune IgG, or NTAb in the presence of excess nitrotyrosine, followed by secondary antibody for staining.

Figure 5 is a bar graph representing the level of myeloperoxidase (MPO) activity from granulocyte infiltration in guinea pig ileum treated with trinitrobenzene sulfonic acid (TNBS) to induce inflammation, and treated with various levels of aminoguanidine.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to antibodies raised against nitrotyrosine which can specifically recognize and bind to nitrotyrosine itself or nitrotyrosine amino acid residues incorporated into larger molecules such as proteins. These antibodies do not crossreact with bovine Cu,Zn superoxide dismutase, bovine serum albumin, keyhole-limpet hemocyanin, lysozyme, histone, actin, catalase, pulmonary surfactant proteins or proteins from entire brain homogenates, but recognize all of these proteins after peroxynitrite treatment. Peroxynitrite treated-human Cu,Zn superoxide dismutase, which contains no tyrosines, does not bind the antibody. Antibody

binding can be blocked by coincubation with 10 mM nitrotyrosine, but not by aminotyrosine or phosphotyrosine. Tissue sections could also be washed with alkaline solutions of sodium dithionite to reduce nitrotyrosine residues to aminotyrosine, which also eliminated antibody binding.

There is very little nitrotyrosine in normal human or animal tissues. The formation of nitrotyrosine is highly correlated with the production of nitric oxide-based oxidants during sepsis, inflammation and ischemia/reperfusion. Addition of nitric oxide under aerobic or anaerobic conditions does not cause significant amounts of tyrosine nitration, indicating that biological detection of nitrotyrosine is a marker of oxidants derived from nitric oxide rather than nitric oxide itself. Thus, one extremely useful application of antinitrosine antibodies is as a marker of oxidative damage occurring to tissue. In addition, nitrotyrosine has been found in a variety of tumors by immunohistochemical staining methods.

The term antibody herein is intended to encompass both polyclonal and monoclonal antibodies. The term antibody is also intended to encompass whole antibodies, biologically functional fragments thereof, chimeric and humanized antibodies comprising portions from more than one species. Also encompassed in the term antibody are antibodies and biologically functional fragments thereof with alterations in glycosylation or with alterations in complement binding function.

The chimeric antibodies can comprise proteins derived from two different species. The portions derived from two different species can be joined together chemically by conventional techniques or can be prepared as a single contiguous protein using genetic engineering techniques (See, e.g., Cabilly et al., U.S. Patent No. 4,816,567, Neuberger, M.S. et al., WO 86/01533 and Winter, G.P., EP 0,239,400). DNA encoding the proteins of both portions of the chimeric antibody can be expressed to produce a contiguous protein. Such engineered antibodies can be, for instance, complementarity determining regions (CDR)-grafted antibodies (Tempest, P.R. *et al.*,

Biotechnology 9:266-271 (1991)) or "hyperchimeric" CDR-grafted antibodies which employ a human-mouse framework sequence chosen by computer modeling (Queen, C. *et al., Proc. Natl. Acad. Sci. USA* 86:10029-10033 (1989)). The constant region domains can be chosen to have an isotype most suitable for the intended application of the antibodies. For example, the human _1 heavy chain appears to be the most effective for complement and cell-mediated killing. The human γ4 heavy chain may be more suitable for imaging, blocking and delivery of therapuetic agents, where complement binding is undesirable (Morrison, S.L. *Ann. Rev. Immunol. 10*:239-265 (1992)).

Biologically functional antibody fragments are those fragments sufficient for binding of the antibody fragment to nitrotyrosine to occur, such as Fab, Fv, $F(ab')_2$, and sFv (single-chain antigen-binding protein) fragments. One can choose among these or whole antibodies for the properties appropriate to a particular method. For example, for a diagnostic application where signal-to-noise ratio or tumor-normal tissue ratio is most important, a radiolabeled sFv may be suitable (Milenic D., *et al., Cancer Res. 51*:6363-6371 (1991)).

In this instance, monoclonal antibodies have been produced by hybridomas (Kearney, J.F. In *Fundamental Immunology*, pp.751-766, Raven Press, New York (1984)). However, monoclonal Fab fragments can also be produced by other methods, for example by using bacteriophage to display and select polypeptide chains expressed from a V-gene library (Hoogenboom, H.R., *et al., Immunol. Rev. 1992*:41-68.

Diagnostic Applications

The nitrotyrosine antibodies can be used to detect nitrotyrosine residues by immunohistochemistry, immunobloting (Western) methodology, by Enzyme-Linked Immuno-Sorbant Assays (ELISA) or radioimmune assays to analyze biopsies, autopsy samples and body fluids. In addition, the antibodies can be used for immunoprecipitation and to concentrate nitrotyrosine-containing proteins from dilute biological solutions for

further analyses. Because the antibodies recognize a small hapten, there is no species specificity, so the antibodies work equally well on human as well as animal samples.

The antibodies can be used, for instance, to examine formalin and glutaraldehyde-fixed, paraffin embedded tissue sections, as well as with frozen sections. The antibodies can thus be used in a variety of surgical biopsies as well as autopsy tissues.

There are several specific controls that can be used to insure the specificity of the antibodies. Antibody binding can be eliminated by coincubation of the nitrotyrosine antibody with 10 mM nitrotyrosine, but not by 10 mM aminotyrosine or phosphotyrosine. The concentration of nitrotyrosine needed to block antibody binding to nitrated bovine serum albumin is between 60-90 μM. Second, the tissue section or protein sample can be treated with 100 mM sodium hydrosulfite in pH 8.5 to 9.0 buffer to reduce nitrotyrosine to aminotyrosine. Chemical reduction eliminates specific binding of nitrotyrosine antibodies.

Other methods can be used to detect the presence of nitrotyrosine. Nitrotyrosine content can be quantified by HPLC analysis of amine-derivatized free amino acids following hydrolysis in 6 N HCl for 16-24 h at 110°C (Haddad, I.Y., *et al., Am. J. Physiol. 265* (Lung Cell Mol Physiol 9):L555-L564 (1993)). Detection of nitrated tyrosine residues by polyclonal antinitrotyrosine antibody in peroxynitrite treated bovine serum albumin correlated with quantitation using a modified HPLC which relies on the intrinsic ultraviolet (UV) absorbance of nitrotyrosine at 280 nm.

It is possible to selectively target reagents to injured or abnormal tissues containing nitrotyrosine by chemically coupling such reagents to antibodies specific to nitrotyrosine and adding the coupled antibody-reagent molecules to a sample of tissue in a diagnostic test. Methods of chemically coupling enzymes, biotin, and various fluorochrome labels to antibodies are known (Harlow, E., *et al., Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988). Other coupling methods

and chemical linkers have been developed more recently (see e.g., Delprino, L. *et al.*, *J. Pharm. Sci.* *82*:699-704 (1993)). These and similar methods can be used to chemically link various therapeutic and diagnostic agents to antibodies specific to nitrotyrosine. Alternatively, various therapeutic and diagnostic agents can be linked to antinitrotyrosine antibodies by biological means, for example, by fusing a gene encoding an enzyme to a gene encoding a chain of an antibody.

Therapeutic Applications

Antinitrotyrosine antibodies can be used in conjugates to deliver therapeutic agents to sites of damage from oxidation. Alternatively, antinitrotyrosine antibodies can be used in conjugates that would bind at a site of damage from oxidation and serve to localize a therapeutic agent in a second step. For example, an enzyme that converts a compound from an inactive to a therapeutically active form can be conjugated to the antibodies. Agents that can be chemically coupled to antinitrotyrosine antibodies and delivered directly, are, for instance, the anti-inflammatory adrenocortical steroids such as hydrocortisone and prednisone.

In a similar manner, agents useful in the treatment of cancer can be delivered to a tumor in an animal or a human, by the use of a conjugated antitumor agent-antinitrosine antibody. Tumor cells have been observed to stain heavily using antinitrotyrosine antibodies in standard histological staining methods. Drugs useful for this application can be, for example, A7-neocarzinostatin (Takahashi, T., *et al.*, *Jpn. J. Cancer Res.* *84*:976-981 (1993)), blocked ricin (Shah, S.A. *et al.*, *Int. J. Immunopharacol.* *15*:723-736 (1993)) and idarubicin (Smyth, M.J., *et al.*, *Immunol. Cell Biol.* *71*:167-179 (1993)).

Single-chain antigen-binding proteins, which are composed of an antibody V_L chain connected to a V_H chain by a peptide linker, have also been used to make immunotoxins. For example, a truncated form of *Pseudomonas* endotoxin has been attached to a F_V that is specific for the IL-2 receptor, and has proven to be highly cytotoxic for IL-2 receptor-bearing cells (Kreitman,

R.J., *et al., Proc. Natl. Acad. Sci. USA* 87:8291-8295 (1990)). Similarly, F_V proteins could be made from antinitrotyrosine antibodies, and a variety of toxins linked to them.

In addition to toxins or other drugs which would act on targets inside a cell exhibiting nitrotyrosine on its surface, various enzymes can be attached to antinitrotyrosine antibodies. Such enzyme-antibody conjugates can act on extracellular substrate as well as intracellular substrate. Examples of enzymes that can be chemically conjugated to antinitrotyrosine antibodies are the antioxidants superoxide dismutase and catalase. These enzymes have as their substrates O_2^- and H_2O_2 respectively.

Bispecific antibodies can be made from monoclonal anitnitrotyrosine antibodies and monoclonal antibodies specific for the CD3 complex, for example. These bispecific antibodies can be used to target T cells to tumor cells (Bohlen, H., *et al., Blood 82*:1803-1812 (1993); Fanger, M.W., *et al., Cancer Treat. Res. 68*:181-194 (1993)).

Antibodies specific for nitrotyrosine can be labeled with a radioactive substance, such as ^{131}I or ^{186}Re, not only for imaging and radioimmunoguided surgery (Martin, E.W., *et al, Am. J. Surgery 156*:386-392 (1988)), but also for cancer therapy (Carrasquillo, J. *Cancer Treat. Res. 68*:65-97 (1993); Gerretsen, M. *et al., Cancer Res. 53*:3524-3529 (1993)). Streptavidin- or avidin-conjugated monoclonal antibodies have been tested in an indirect method useful for imaging or localized therapy in which a radiolabeled derivative of biotin was added and assayed for its specific localization at the sites of antibody binding (Rosebrough, S.F., *Nucl. Med. Biol. 20*:663-668 (1993)). Antinitrotyrosine antibodies can be used in a similar manner with a streptavidin-biotin labeling system.

Nitration of tyrosine residues may make proteins more antigenic and cause or contribute to autoimmune disorders, such as ankylosing spondylitis, systemic lupus erythematosus and multiple sclerosis. Nitrobenzene derivatives are highly antigenic. It has long been known that proteins can be made antigenic by coupling them to small organic molecules (haptens)

such as dinitrophenol and aminobenzoic acid (Landsteiner, K. *The Specificity of Serologic Reactions.* Dover Press, 1962).

Antibodies to nitrotyrosine may be administered to a patient to block the binding of endogenously produced autoantibodies to nitrotyrosine. Alternatively, peptides or proteins containing aminotyrosine can be administered to a patient who produces antinitrotyrosine antibodies, to bind to those antibodies, thereby preventing them from binding to tissues.

Antibodies to nitrotyrosine may also be used as interference proteins to study the effects of endogenously produced autoantibodies on the performance of clinical immunoassays for both antigens and antibodies. Artificially produced human antibodies that mimic autoantibody reactivies can be added to human sera containing a defined quantity of nitrotyrosine. By varying the concentrations of the ligand and the interfering autoantibody, one can examine the extent to which the results of a clinical immunoassay are altered (Hamilton, R.G., *et al., Clin. Chem. 39*:1988-1997 (1993)).

Antibodies to nitrotyrosine to be used in methods of therapy may be administered by a number of routes, including, for example, intramuscularly, intravenously, intraperitoneally, or enterally, or by aerosal, using preparations of composition appropriate to the route of delvery.

Disease States Relevant to Applications for Antibodies

Levels of tyrosine nitration are very low to undetectable in normal lung, but increase rapidly in response to sepsis and inflammation. Adult Respiratory Distress Syndrome (ARDS), also known as shock lung, is the leading cause of death in intensive care units, but is hard to diagnose until the disease is well established. In active ARDS, essentially all tissues can be extensively nitrated, though staining with anitnitrotyrosine antibody is particularly intense in and around inflammatory cells. In one surviving ARDS patient, a biopsy taken from the lung a year later showed no significant nitration. Bronchial

alveolar lavages and small biopsies removed by endoscopy can be stained for nitrotyrosine to assess cell damage from oxidants.

Nitric oxide is now being widely administered to patients with pulmonary hypertension and also with active inflammation. The vasorelaxing properties of nitric oxide, which are mimicked clinically with nitroglycerin and sodium nitroprusside, dilate pulmonary blood vessels and reduce the work necessary for the heart to pump blood through the lungs. However, the nitric oxide may also react with superoxide produced by pulmonary inflammatory cells to form peroxynitrite and nitrate proteins in the lung. The development of this adverse reaction can be followed by monitoring nitration of proteins in lung lavages and aspirates using antibodies to nitrotyrosine.

Ischemia is known to stimulate nitric oxide production in brain to remarkable concentrations. Nitric oxide production is initiated by increased intracellular calcium, which is known to be greatly elevated during ischemia by activation of glutamate-receptor-controlled calcium channels. The production of nitric oxide has been implicated in ischemic neuronal death (Dawson, V.L. *et al.*, *Proc. Natl. Acad. Sci. USA, 88*:6368-6371 (1991); Dawson, V. *et al.*, *Proc. Natl. Acad. Sci. USA, 90*:3256-3259 (1991)) and infarction (Nowicki, J.P. *et al.*, *Eur. J. Pharmacol., 204*:339-340 (1991)). Nitrotyrosine binds with high affinity to infarct bound tissue in a model of focal cerebral ischemia. It has been shown previously that an antioxidant enzyme, polyethylene glycol conjugated superoxide dismutase, is protective in this stroke model (Liu, T.H. *et al.*, *Am. J. Physiol., 256*:H589-H593 (1989)), which has led to successful clinical trials (Muizelaar, J. *et al.*, *J. Neurosurg, 78*:375-382 (1993)). In human brain, the antibodies heavily stain Hirano bodies, which are unusual structures containing large amounts of actin and actin binding proteins.

Ileitis, or inflammation of the bowel, is a major clinical problem, causing 8% of thoracic surgical deaths. It also is highly correlated with the development of colon cancer. In a standard rat model of ileitis involving the injection of a nitrobenzene derivative into the colon wall, there is a large

deposition of nitrotyrosine in degenerative epithelium. It is highly correlated with the induction of nitric oxide synthase. Furthermore, inhibition of nitric oxide production was highly protective and prevented the formation of nitrotyrosine.

Demonstration of Nitrotyrosine in Atherosclerosis

Two human atherosclerotic coronary arteries with atherosclerotic narrowing of the vessel lumen from patients who died of coronary disease at ages 57 and 63 were examined. (Beckman, J.S. *et al. Biol. Chem. Hoppe-Seyler 375*:81-85 (1994) is hereby incorporated by reference). The coronary artery from the first patient shows substantial subintimal accumulation of lipid and cholesterol with foamy cell infiltrate with a fibrous cap beneath the endothelium. There is a large eccentric atherosclerotic plaque containing a central necrotic core, cholesterol clefts, lipid-laden macrophages around the perphery and a fibrous cap. Immunoperoxidase stained serial sections showed binding of the nitrotyosine polyclonal antibody (1 to 500 dilution; Example 1). Brown reaction product indicated specific antibody binding. Staining was pronounced around the atherosclerotic plaque, especially around the periphery in areas where macrophages accumulated, and in the subepithelial region of the vessel wall. The fibrous cap showed little staining. Staining with pre-immune serum instead of the primary antibody was performed to serve as a negative control. The trichrome-stained section of coronary artery from the second patient exhibited substantial narrowing of the lumen and evidence of hemorrhage through a rupture of the fibrous cap. A serial section demonstrated binding of the monoclonal nitrotyrosine antibody (1 to 10) against nitrotyrosine. The brown reaction product was concentrated in the atherosclerotic plaque and in the subendothelial areas of the vessel lumen. Staining with the same concentration of antibody blocked with 10 mM nitrotyrosine served as a negative control.

Extensive immunoreactivity with nitrotyrosine-containing proteins was observed around the atheroma in both coronary arteries. High power magnification of these arteries revealed

cholesterol crystals and accumulations of lipid laden macrophages (foam cells) in the atherosclerotic lesions. Serial sections demonstrated binding of the polyclonal nitrotyrosine antibody (1 to 500 dilution) against nitrotyrosine. The brown reaction product was concentrated in foam cells. Endothelium also stained heavily and did not appear to be an edge effect because the distribution was distinctly cellular and in the same focal plane of the endothelial cells. Fibrotic regions and red blood cells did not significantly bind the antibody. Staining was less pronounced in the hemorrhagic region of the plaque. However, macrophages entrapped in the hemorrhage were strongly stained. Nuclei tended to be stained more heavily, even in cells with relatively low binding of the nitrotyrosine antibodies. Nuclear staining was not present in pre-immune controls or in controls where the antibody was coincubated with 10 mM nitrotyrosine, suggesting nitrotyrosine-containing proteins may be found in nuclei. No staining was observed away from the inflammatory cells.

The binding of monoclonal and polyclonal antibodies to tissue was blocked by co-incubation with 10 mM nitrotyrosine. Equivalent concentrations of aminotyrosine or phosphotyrosine adjusted to pH 7.4 did not block the binding of the nitrotyrosine antibodies. No binding was observed with control rabbit or mouse IgG replacing the primary antibody. Pretreatment of the sections with alkaline sodium hydrosulfite also prevented antibody binding in the tissue sections, though it tended to slightly disrupt the preservation of the tissue structure. No staining was observed away from the inflammatory cells. Immunoreactivity for nitrotyrosine was also evident in inflammatory cells in a fatty streak found in the subintimal layer of the aorta (section stained with trichrome) from a 34 yr old male who died in a traffic accident. In a serial section reacted with the monoclonal antibody (1 to 10 dilution), brown staining was concentrated in foam cells of the fatty streak and diffuse staining with the subintima.

Neither the polyclonal nor the monoclonal antibodies showed cross reactivity with either mouse, rabbit or bovine serum albumin, or with rat heart or brain homogenates in western blots (Figure 1). There was a slight labeling of several high molecular mass bands in heart homogenate, which may be due to endogenous nitration. The antibody did not cross react with any of these protein preparations exposed to 5 mM hypochlorous acid or xanthine oxidase plus Fe^{3+}EDTA (Figure 1).

Evidence for *In Vivo* Peroxynitrite Production in Human Acute Lung Injury

Human lung sections were immunohistochemically stained utilizing specific antibodies that recognize nitrotyrosine residues (Example 1). The polyclonal antibody was utilized in a dilution of 1 to 200, and the monoclonal antibody was utilized in a dilution of 1 to 10 in 0.05 M Tris-HCl/0.15 M sodium chloride buffer, pH 7.4. Diaminobenzidene-peroxidase reaction results in brown staining in the presence of nitrotyrosine residues. In all tissue specimens from patients with diffuse alveolar damage (Example 2), immunohistochemical staining of protein nitrotyrosine residues was observed throughout the lung, including the lung interstitium, alveolar epithelium, proteinaceous alveolar exudate, and alveolar macrophages. In addition, patients with sepsis-induced diffuse alveolar damage had staining of the vascular endothelium and subendothelial tissue. Diffuse alveolar damage from nonseptic insults did not result in vascular nitrotyrosine formation. However, staining was noted within vascular thrombi in these patients. Extensive staining of the intra-alveolar exudate and neutrophils was observed in the tissue specimens from the patient with bacterial bronchopneumonia, but the lung parenchyma and vascular structures had no nitrotyrosine residues present. Minimal staining of the alveolar septum only was observed in control lung specimens. These findings are summarized in Table 1.

The binding of monoclonal and polyclonal antibodies to tissue was blocked by coincubation with 10 mM nitrotyrosine and

1 mg/ml nitro-bovine serum albumin. Aminotyrosine (10 mM), phosphotyrosine (10 mM), or bovine serum albumin (1 mg/ml) did not block the binding of the nitrotyrosine antibodies. Pretreatment of the tissue specimens with sodium hydrosulfite at pH 9.0 to 9.5 to reduce nitrotyrosine to aminotyrosine prevented antibody binding to the tissue samples.

Immunohistochemical staining of human lung tissue with antibody to nitrotyrosine appears to correlate with the mechanism and severity of lung injury. Patient 1 had overwhelming septic shock and died in the early exudative phase of acute lung injury. Lung tissue specimens from this patient were the most intensely stained, and included distinct staining of the pulmonary interstitium, pulmonary epithelium, inflammatory cells, intraalveolar proteinaceous exudate, and vascular endothelium and subendothelium. Patient 2, also with sepsis, was not as acutely ill and died during the later fibroproliferative stage of acute lung injury. Tissue specimens from this patient were not as intensely stained, but staining of specific tissue elements was consistent with the staining seen in patient 1. The presence of nitrotyrosine residues within the vascular structures as well as the lung interstitium and alveolar space is consistent with sepsis-induced acute lung injury being a manifestation of diffuse intravascular inflammation. Acute lung injury associated with nonseptic insults resulted in nitrotyrosine formation in the lung interstitium and alveolar space, but lacked the presence of nitrotyrosine residues in the pulmonary vascular bed, consistent with a non-systemic lung insult. Nitration of tyrosine residues present in the inflammatory cells and intra-alveolar proteinaceous exudate, but not in the pulmonary interstitium or the vascular structures in the patient with bacterial bronchopneumaonia, is consistent with bronchopneumonia being an intra-alveolar inflammatory process.

TABLE 1: CLINICAL SUMMARY OF STUDY PATIENTS

Pat.	AGE AT ONSET OF ACUTE ILLNESS	DURATION OF ACUTE ILLNESS	DIFFUSE ALVEOLAR DAMAGE	P_aO_2/FiO_2	OTHER DIAG.
1	6 WEEKS	4 DAYS	YES	130	SEPTIC SHOCK DISSEM. INTRAVASC. COAGULATION
2	3 YEARS	19 DAYS	YES	65	IMMUNOSUPPRESSION SEPTIC SHOCK
3	3 MONTHS	2 DAYS	YES	92	S/P CARDIOPULMONARY ARREST CARDIOGENIC SHOCK
4	1 DAY	12 DAYS	YES	36	MECONIUM ASPIRATION PERSISTENT PULMONARY HYPERTENSION
5	3 WEEKS	1 DAY	NO	37	ACUTE BRONCHOPNEUM. SEPTIC SHOCK
6	3 MONTHS	3 DAYS	NO	619	GLYCOGEN STORAGE DISEASE SEIZURES ISCHEMIC ENCEPHALOPATHY

Quantitation of Nitrotyrosine Levels in Lung Sections of Patients and Animals With Acute Lung Injury

Paraffin embedded sections of lung (Example 3) from a patient with sepsis-induced ARDS (A.P.; see Table 2) were deparaffinized, fixed in methanol, and permeabilized as described in Example 4. Sections were then incubated with non-immune IgG, NTAb (polyclonal antibody raised against nitrotyrosine; see Example 1), or NTAb in the presence of excess nitrotyrosine (10 mM). All sections were then incubated with a secondary antibody coupled to rhodamine. Using a fluorescent microscope, four random images were captured by a camera, and entered into a computer for immunofluorescence quantification. All pictures were obtained with identical camera and computer settings (Example 4). Immunostaining was reduced to control levels when the lung section was incubated with the NTAb in the presence of an excess amount of antigen (10 nM nitrotyrosine), or when the primary antibody was replaced with an equal amount of non-immune IgG. Figure 2 shows the relative distribution of the mean fluorescence intensity among the different lung fields examined from another ARDS patient (C.D.; see Table 2). Staining was continuous and uniform around the blood gas barrier.

Frozen lung sections from a subject who died without clinical or pathologic evidence of acute lung inflammation (patient T.H.) were also examined as a control. No significant difference was detected between the recorded fluorescence values obtained when tissues were immunostained with the polyclonal NTAb and when the antibody was substituted with an equivalent amount of non-immune IgG.

For each patient, values for the various fields (at least n = 4) were averaged and the mean value was used to calculate the group mean (Figure 3). Thus, each patient was weighted equally in the calculation of the group mean. All pictures were obtained with identical camera and computer settings. Sections from ARDS lungs incubated with the NTAb exhibited approximately two times the fluorescence of those incubated with non-immune IgG or sections of control lungs incubated with

the NTAb. Values are means ± SEM. Statistical differences among group means were determined using one way analysis of variance and the Bonferonni modification of the t-test. (* = P < 0.05 compared with the corresponding IgG value.) These results indicate that the lungs of patients with clinical and pathologic ARDS, but not lungs without evidence of acute or chronic inflammation, contained significant levels of residues antigenically related to nitrotyrosine. Fluorescence was blocked in the presence of 10 mM nitrotyrosine, suggesting the excess antigen reacted with the NTAb and prevented binding to tissues.

Rats were exposed to 100% O_2 for 60 hours (Example 5), which results in a form of lung injury that has many of the characteristics of human ARDS. Lung sections from exposed (n=3) and control (exposed to room air) rats were treated with NTAb, an equivalent amount of non-immune IgG, or NTAb in the presence of excess nitrotyrosine, followed by secondary antibody coupled to rhodamine. All pictures were obtained with identical camera and computer settings. For each rat, values for the various images (at least n = 4) were averaged and the mean value was used to calculate the group mean. All pictures were obtained with identical camera and computer settings. Sections from hyperoxic rat lungs incubated with the NTAb exhibited approximately two times the fluorescence compared with those incubated with non-immune IgG or sections of control lungs incubated with the NTAb. Values are means ± SEM. (* = P < 0.05 compared with corresponding IgG value.)

As found in the lung sections of patients with severe acute lung injury, immunostaining was totally absent when lung sections were incubated with both the polyclonal antibody in the presence of nitrotyrosine (10 mM), or with an equivalent amount of non-immune IgG. Mean fluorescence values for the hyperoxic and control groups are shown in Figure 4. Data shown in this figure indicate the presence of residues antigenically related to nitrotyrosine in the lungs of rats exposed to hyperoxia.

Rat lung sections were treated with peroxynitrite (1mM) followed by incubation with the NTAb (Example 6). Lung sections were dipped in 10 mM HEPES buffer, pH 7.4 during exposure to oxidants. Significant levels of nitrotyrosine were demonstrated in the lung sections incubated with synthetic peroxynitrite (1 mM) but not in those incubated with hydrogen peroxide, superoxide anions and hydroxyl radicals, generated by the action of xanthine oxidase (10 mM) on pterine (2-amino-4-hydroxypteridine) (200 μM), or with NO alone, generated by SNAP (SNAP = S-nitroso-N-acetylpenicillamine; 100 μM plus 100 μM L-cysteine).

TABLE 2. Characteristics of ARDS Patients

Patient	Age/Sex	Diagnosis	Clinical Phase	Lung Pathology
1) C.D. autopsy	14 yr/F	lymphoma	Late	f.f. & par./ fibro-proliferative
2) A.P. autopsy	9 yr/F	sepsis	A.R.F.	par./cellular proliferative
3) A.R. autopsy	3 m/M	sepsis	A.R.F.	par/exudative
4) T.H. autopsy	23 m/F	near drowning	A.R.F.	par./cellular proliferative
5) J.J. biopsy	19 m/M	upper airway obstruction	Late	par./fibro-proliferative

Definition of abbreviations: M = male; F = female; m = months; yr = years; A.R.F. = acute respiratory failure; f.f. = fresh frozen; par. = paraffin embedded.

TABLE 3. Characteristics of non-ARDS Patients

Patient	Age/Sex	Diagnosis	Pathology
6) T.H. autopsy	12 yr/F	M.D./heart failure	f.f./ lung edema
7) C.M. autopsy	2 w/M	I.V.H.	f.f./normal
8) C.H. autopsy	1 m/F	sepsis	f.f./ lung edema
9) M.C. autopsy	14 yr/M	Marfan's/ cardiac tamponade	f.f./normal
10) J.J. biopsy	3 yr/M	Upper airway obstruction	par/ consistent with healed B.P.D.

Definition of abbreviations: M = male; F = female; m = months; yr = years; A.R.F. = acute respiratory failure; f.f. = fresh frozen; par = paraffin; M.D. = muscular dystrophy; I.V.H. = intra-ventricular hemorrhage; B.P.D. = bronchopulmonary dysplasia.

Inducible NOS Gene Expression

RNA extracts of ileum from control or TNBS-treated guinea pigs (TNBS is trinitrobenzene sulfonic acid; Example 7) were subjected to reverse transcriptase polymerase chain reaction (RT-PCR) using a 907 base pair probe to identify iNOS gene expression (Example 8). We found iNOS gene expression in control animals to be faint to undetectable with this technique. In contrast, iNOS transcription was readily demonstrable in TNBS-treated animals throughout the time-course of the study, days 1-7. This persistent tissue expression of iNOS may reflect continued expression in individual cells, but more likely reflects the activation of iNOS gene expression in infiltrating or newly generated cells in the gut wall.

Immunohistochemistry for Nitric Oxide Synthase and Nitrotyrosine

The non-specific histochemical stain for NOS, NADPH diaphorase, and immunohistochemical localization of iNOS and nitrotyrosine were compared (Example 7). In control guinea pigs, NADPH diaphorase staining was confined to neurons in the myenteric and submucosal plexuses. In TNBS-treated animals, NADPH diaphorase positive staining was also evident in epithelial cells and the mucosal lamina propria. In order to ascertain if NADPH diaphorase positive cells were expressing iNOS, we used an antibody raised to a conserved region of mouse iNOS. In control animals, immunoreactive iNOS was largely absent although some positive staining (brown color) was evident in villus tips. In TNBS animals at day 7, marked NADPH diaphorase staining was evident in the mucosa, including lamina propria and the epithelium. Staining in the crypts was negligible in comparison with the villus tips as staining intensity increased along the length of the villus. In TNBS-treated guinea pigs the localization of immunoreactive iNOS was similar to NADPH diaphorase, being particularly pronounced in terminal epithelia. In myenteric ganglia of TNBS-treated guinea pigs, extensive DAB (3,3'-diaminobenzidine) peroxidase staining was observed in neurons for both iNOS and

nitrotyrosine of TNBS-treated animals. In control animals, staining was very weak for iNOS and negligible for nitrotyrosine. Submucosal ganglia were also positive for iNOS and nitrotyrosine in TNBS ileitis.

Strong staining for NADPH diaphorase, iNOS and nitrotyrosine demonstrated the co-localization of iNOS and nitrotyrosine in terminal epithelial cells and myenteric ganglia of TNBS-treated animals. Epithelial cells being extruded into the gut lumen were particularly well stained, raising the potential that iNOS and peroxynitrite-based mechanisms contribute to epithelial apoptosis. Neurons in the myenteric ganglia of TNBS animals were also noticeably stained for both nitrotyrosine and iNOS. Nerve fibers extending into the muscularis externa were also positively stained.

To evaluate the role of peroxynitrite in this model, the location of nitrotyrosine in the TNBS animals with and without concurrent treatment with the NOS inhibitor, L-NAME, was assessed by immunohistochemistry.

L-NAME treatment corrected the disturbances in morphology associated with TNBS administration. In TNBS-alone treated animals, extensive staining was evident throughout the gut wall, but particularly in the epithelia and myenteric ganglia. In TNBS + L-NAME treated animals, nitrotyrosine immunoreactivity was absent. When antibody to nitrotyrosine was preincubated with exogenous nitrotyrosine prior to tissue application, this eliminated the positive tissue staining due to TNBS ileitis. Staining in TNBS + L-NAME tissue remained negligible, confirming that L-NAME treatment prevented nitrotyrosine formation in TNBS ileitis.

Effects of Aminoguanidine Administration

Inclusion of aminoguanidine in the drinking water after TNBS administration resulted in a dose-dependent inhibition of the inflammatory response. All morphological and functional indices examined were corrected by aminoguanidine. Granulocyte infiltration, quantified with the MPO (myeloperoxidase) assay, was marked 7 days after TNBS administration. Aminoguanidine

dose-dependently prevented granulocyte infiltration in TNBS animals at doses of 1-10 µg/ml (Figure 5). At lower doses aminoguanidine was less effective, with no statistical determination of protection at 10 ng/ml. The EC_{50} for preventing the MPO response was 100 ng/ml, which approximates 13 µg/kg/day based on fluid consumption. Aminoguanidine had no effect on basal MPO values on intact guinea pigs.

Aminoguanidine prevented the increase in ileal thickness (determined from the ratio of weight per length) induced by TNBS administration (Table 4). Ileal lavages were used as indices of localized production of nitric oxide. Lavage nitrite levels were markedly elevated in TNBS ileitis; aminoguanidine resulted in the restoration of lavage nitrite levels to normal. Aminoguanidine also prevented the increase in lavage protein level which accompanied TNBS-induced gut inflammation (Table 4).

TABLE 4. Ileal Weight, Lavage Protein and Nitrite Levels in TNBS Ileitis

	SHAM	SHAM + AG	TNBS	TNBS + AG
Ileal Weight mg/cm	70±3 (11)	75±5 (15)	94±5** (19)	77±4 (11)
Lavage Protein µg/g	145±26 (10)	233±72 (5)	436±74** (19)	191±30 (10)
Lavage Nitrite nmol/g	30±4 (10)	24±3 (5)	63±9* (19)	39±8 (10)

Results expressed as mean _ SEM.
The number of observations for each group are in parenthesis.
* p <0.05 vs. all other groups
** p <0.01 vs. all other groups.

Example 1: Preparation of antibodies and their use in staining coronary arteries

Peroxynitrite was prepared from quenching the reaction of acidified nitrate and hydrogen peroxide with sodium hydroxide as previously described (Beckman, J.S. *et al. Arch. Biochem. Biophys. 298*:438-445 (1992)). Rabbits were injected with peroxynitrite-modified keyhole limpet hemocyanin (Pierce Chem. Co., Rockville, Ill.). Keyhole limpet hemocyanin (8 mg) in 5 ml of 100 mM potassium phosphate (pH 7.4) plus 1 mM Fe^{3+}EDTA was mixed rapidly with 2 mM peroxynitrite (final concentration). After dialysis overnight, the protein was mixed with an equal volume of Freund's complete adjuvent to a final concentration of 0.5 mg/ml. Rabbits were boosted biweekly with 0.5 mg nitrated keyhole limpet hemocyanin mixed with an equal volume of Freund's incomplete adjuvent. IgG was purified with "Gamma Bind G" affinity columns from Pharmacia and dialyzed against phosphate buffed saline overnight. The rabbit polyclonal antibody was used in dilutions of 1 to 200 for immunohistology, and was effective at detecting peroxynitrite modified bovine serum albumin at dilutions of 1 to 256,000.

Mice (BALB/C, Charles River Breeding Laboratories) were immunized with peroxynitrite-modified keyhole limpet hemocyanin according to Lieberman *et al.*, (*J. Exp. Med. 142*:106-119 (1975)) with slight modification. The primary immunization was 1 mg/ml of nitrated keyhole limpet hemocyanin in phosphate buffered saline emulsified with an equal volume of Freund's complete adjuvent (Gibco Laboratories). Each mouse received 0.3 ml of the vaccine delivered subcutaneously in the rear hind foot pads and over the abdominal area. Seven days later the mice were injected in a similar manner with 100 µg nitrated keyhole limpet hemocyanin in phosphate buffered saline alone. Approximately sixteen days after the first immunization, the spleen and popliteal lymph nodes were removed, prepared as a cell suspension and fused with the non-secreting cell line P3X63-Ag8.653 as described by Kearney (In: *Fundamental Immunology*, Raven Press, New York (1984)). After fusion, cells

were seeded into 24 well tissue culture plates (Costar) in plating media consisting of RPMI 1640 media with 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 µg streptomycin and HAT (hypoxanthine, aminopterin and thymidine medium; ICN-Flow Laboratories). Fusions were fed on days 7 and 9 by replacing half of the spent media with fresh HAT media. The last feeding, 48 hr prior to screening, consisted of a complete change of media. On day 14 after fusion, supernatants were screened for the combination of binding to peroxynitrite-modified bovine serum albumin, the lack of binding to bovine serum albumin and blockage of binding by 10 mM nitrotyrosine by ELISA. Positive wells were cloned by limited diffusion on feeder cells obtained from the peritoneal cavity of pristane-primed mice. Hybridoma cell lines were raised as ascots in pristane-primed BALB/C mice. A suitable colony was used for preparing ascites fluid and the IgG fraction by Protein A chromatography was purified for use in subsequent studies at dilutions of 1/10 to 1/100.

Human coronary arteries were collected at autopsy, preserved in formalin and embedded in paraffin. The immunostaining utilized the DAKO avidin-peroxidase kit, with diaminobenzidine tablets from Sigma substituted at the development stage. Sections were counterstained with hematoxylin/eosin.

Two sets of control reactions were run on tissue samples to confirm the presence of nitrotyrosine. Just prior to the addition of the primary antibody to the sections, the antibody was incubated with 10 mM nitrotyrosine in potassium phosphate buffer, pH 7.4. Another section was flooded with three washes of 1 M sodium hydrosulfite for twenty seconds each, to reduce nitrotyrosine to aminotyrosine, and washed extensively with distilled water. The sodium hydrosulfite was prepared immediately before use and the pH adjusted to 9 to 9.5 with sodium hydroxide. It is sensitive to oxygen and was kept in a sealed vacutainer tube and withdrawn in a syringe.

Example 2: Tissue specimens

Autopsies performed at The Children's Hospital of Alabama were reviewed for a pathological diagnosis of diffuse alveolar damage. Formalin-fixed, paraffin-embedded lung tissue samples were obtained from four patients with diffuse alveolar damage, one patient with bacterial pneumonia and shock without diffuse alveolar damage, and one patient without clinical pulmonary disease. A brief clinical summary is provided in Table 1. Eight micron sections of lung tissue were mounted on poly-L-lysine-coated microscope slides. Tissue sections were immunostained using the DAKO avidin-peroxidase kit, with diaminobenzidine tablets (Sigma, St. Louis, MO) substituted at the development stage. Sections were counterstained with Mayer's hematoxylin and eosin (Sigma).

To confirm the specificity of the antibody for nitrotyrosine, just prior to the addition of the primary antibody to tissue samples, the antibody was incubated with 10 mM nitrotyrosine, 1 mg/ml nitrated bovine serum albumin, 10 mM aminotyrosine, 10 mM phosphotyrosine, or 1 mg/ml bovine serum albumin in 0.05 M Tris-HCl/0.15 M NaCl buffer, pH 7.4. Nitrated bovine serum albumin was prepared by reacting bovine serum albumin (Sigma) plus 1 mM $Fe3^{+}EDTA$ in 50 mM potassium phosphate, pH 7.4, with 1 mM peroxynitrite and dialyzing against phosphate buffered saline. Other sections were treated with 1 M sodium hydrosulfite for 1 minute to reduce tissue nitrotyrosine to aminotyrosine. Sodium hydrosulfite was prepared immediately before use in 0.05 M Tris-HCl/0.15 M NaCl buffer and the pH was adjusted to 9 to 9.5 with sodium hydroxide.

Example 3: Human lung section

Lung tissue samples from five pediatric patients with clinical diagnosis of ARDS (age 3 months to 14 years), triggered by a variety of predisposing factors, were obtained either during autopsy (n = 4) or during surgical biopsy (n = 1; to rule out infection). Lung tissue samples were also obtained during autopsy (n = 4) or surgical biopsy (n = 1) from the

lungs of five non-ARDS patients without histologic evidence of pulmonary inflammation. Characteristics of these patients and their primary diagnosis is shown in Tables 2 and 3. All lung specimens were either immediately frozen at -80 °C, or formalin fixed and paraffin embedded. Cryo-sections (6 μM) were cut using a Tissue-Tek II cryostat (Miles; Model 4553) at -30 °C and mounted on glass slides.

Example 4: Immunofluorescence measurements

Paraffin embedded sections were deparaffinized by dipping in successive solutions containing alcohol and xylene. These sections, along with the fresh frozen ones, were then fixed and permeabilized by immersion in 100% methanol at -20 °C for 7 minutes, followed by immersion in 1% cold (4 °C) bovine serum albumin in phosphate buffered saline (PBS) for 3 hours to block non-antigenic sites. They were then incubated with a polyclonal antibody raised against nitrotyrosine (NTAb; 1:200 dilution), followed by a secondary antibody, goat anti-rabbit IgG conjugated to rhodamine (1:300 dilution). The NTAb was raised in rabbits by injecting them with peroxynitrite treated Keyhole Limpet hemocyanin (LKH, Pierce, Rockford, IL), as described in Example 1. In control measurements, tissues were incubated with the NTAb in the presence of 10 mM nitrotyrosine, or with a similar amount of non-immune anti-rabbit IgG. Tissues were then washed with cold PBS to remove unbound antibody, overlaid with a drop of glycerol/PBS (9:1) mounting medium containing 0.01% phenylene diamine to prevent fluorescence bleaching, covered with a coverslip and sealed with Cytoseal 60 Mounting Medium (Stephens Scientific, Denville, NJ). The slides were stored at -20 °C until examined.

Lung sections were viewed with a AusJena Sedival inverted microscope equipped with 50 X planachromat objective, Hoffman optics and fluorescence capability with standard fluorescein (485 ± 20 nm excitation filter; 520-560 nm emission filter) and rhodamine (546 ± 10 nm excitation filter; 590 nm emission filter) filters. For each slide, at least four images were

selected randomly while viewing the slides under light microscopy, and the corresponding fluorescent images were captured by a Photometrics Series 200 cooled charged-coupled device (CCD) camera system connected to Macintosh IIci computer. Video images were digitized into two dimensional arrays of picture elements (pixels). Each pixel is a square with a width of 0.21 microns. In each field of view (500 magnification), there are 221,184 pixels corresponding to 9754 sq. microns (23 pixels/sq. micron). Each pixel was assigned an intensity value ranging from 0 (black) to 16384 (white). Alveolar structures in each image were outlined using digital imaging techniques. The intensity value of each pixel within the outlined area was measured and a mean intensity was calculated using the IPLab Spectrum 2.2.1 software (Signal Analytics Co., Vierna). Background intensity was digitally subtracted, as previously described (Matalon, S. *et al.*, *Am. J. Physiol.*, *262*:C1228-C1238 (1992)).

Example 5: Exposure of rats to hyperoxia

Adult male Sprague-Dawley rats (275-350 g) were exposed to 100% O_2 for 60 hours inside specially designed environmental chambers constructed from Lucite®, with a metal top to facilitate heat transfer. Throughout the exposure period, the chambers were housed in a room kept at a constant 21 °C. The construction of the chambers allowed for the administration of food and water to the rats and the removal of animal waste through airlocks without altering the gas composition of their environment. The chambers were continuously flushed with 10 l/min of 100% O_2 (Airco, Birmingham). The oxygen concentration in the chamber, monitored periodically with an oxygen analyzer (Ohmeda 5120), was always higher than 97%. In all experiments the temperature in the chamber was between 23-24 °C.

At the end of the 60 hour exposure period, the rats were removed from the chambers and anesthetized with an intraperitoneal injection of sodium pentothal (50 mg/kg). The trachea was exposed and a plastic catheter was inserted so its

tip was just above the carina. The catheter was occluded and the lungs were allowed to collapse by absorption atelectasis. The chest was then opened, and ten ml of Tissue-Tek® O.C.T. Compound were instilled into the lungs via a syringe. The lungs and heart were removed from the thoracic cavity *en block*, and immediately frozen at -20 °C. Cryo-sections (6 µM) were cut using a Tissue-Tek II cryostat (Miles; Model 4553) at -30 °C and mounted on glass slides.

Example 6: In vitro exposure of rat lung sections to oxidants

To determine which oxidant is capable of nitrating lung tyrosine residues, lung sections from control rat, prepared as described above in Example 5, were immersed in a 10 nm HEPES solution (150 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1.2 mM $MgCl_2$; pH 7.4). They were then incubated for 2 hours at 37 °C with: (i) a bolus of synthetic peroxynitrite (1 mM), synthesized from sodium nitrite and acidified hydrogen peroxide as previously described (Beckman, J.S. *et al.*, *Proc. Natl. Acad. Sci. USA*, *87*:1620-1624 (1990)); (ii) S-nitroso-N-acetylpenicillamine (SNAP; 100 µM plus 100 µM L-cysteine), which generates a mean NO concentration of about 1 µM over the exposure period (equivalent to 25 ppm of NO in the gas phase); or (iii) xanthine oxidase (10 mU/ml) plus pterine (200 mM) in the presence of 100 µM Fe^{3+}EDTA, which generate superoxide, hydrogen peroxide and hydroxyl radicals. Xanthine oxidase was equilibrated with 10 mM HEPES buffer (pH 7.4). Its activity was determined spectrophotometrically by measuring the rate of urate production at 295 nm in 500 µM xanthine (µM = 1.1×10^{-4} $M^{-1}cm^{-1}$).

Example 7: TNBS treatment of animals and analyses of NO production

Fasted Hartley guinea pigs of either sex (250-450 g) were anesthetized by intramuscular injection of ketamine (40 mg/kg), xylazine (10 mg/kg) and atropine (mg/kg) supplemented with inhaled methoxyflurane as required. Under aseptic surgical conditions a midline laparotomy was performed. The distal

ileum was isolated and gently flushed with intraluminal saline (2 ml) to remove residual enteric contents. Trinitrobenzene sulfonic acid (TNBS, 30 mg/kg) in ethanol (50%, 0.5 ml) was injected transmurally into the lumen with a 26 gauge needle. The peritoneum was closed by suturing layer by layer. The guinea pig was transferred to a warm waterbed for post-operative recovery. Sham animals received the same surgical procedure but received saline instead of ethanol and TNBS.

After 7 days the guinea pigs were anesthetized as described above. After flushing the ileal contents with warm saline, a 10 cm loop of ileum encompassing the region of TNBS administration was made with silk ligatures. Saline (2 ml) was injected into the loop. After 30 minutes the loop was removed from the animal and the loop fluid volume was recorded. After centrifugation, the contents were aliquoted and frozen. Ileal thickness was determined from the ratio of weight to length. Tissue water content and sections were collected for MPO content. Ileum was either frozen in O.C.T. in a dry ice/ethanol bath or placed in either 4% paraformaldehyde or Bouin's fixative for routine histology and immunohistochemistry. Before the animals were killed by anesthesia overdose, cardiac blood was drawn for quantification of peripheral leukocyte counts. All protocols were reviewed and approved by the institutional animal care and use committee, following NIH guidelines and in accordance with the Declaration of Helsinki.

Loop fluid from ileal lavages was analyzed for protein content. Levels of reactive nitrogen intermediates (nitrite/nitrate) were quantified in lavage fluid as an index of local production of NO. Two methods were used, the Greiss reaction (Miller, M.J.S. *et al.*, *J. Pharmacol. Exp. Therapy, 264*:11-16 (1993)) after conversion of nitrate to nitrite with bacterial-derived nitrate reductase (Sigma Chemical Co., St. Louis, MO), or the fluorometric assay described by Misko and colleagues (Misko, T.P. *et al.*, *Analytical Biochemisty, 241*:11-16 (1993)). While the two methods gave quantitatively different results, the trends associated with treatments were

comparable. A Bio-Rad model 3550 plate reader was used for protein and Greiss reaction assays as previously described (Miller, M.J.S. *et al., J. Pharmacol. Exp. Therapy, 264*:11-16 (1993)).

Ileal MPO content was used as an index of tissue granulocyte content. Briefly, ileal mucosal scrapings (100-250 mg) were finely minced at 4°C, homogenized with a Brinkman polytron for 20 s in 50 mM hexadecyltrimethylammonium bromide to inhibit the pseudoperoxidase activity of hemoglobin. The homogenate was centrifuged at 20,000 g for 20 minutes at 4_C and the pellet was then frozen and thawed. This cycle of homogenization, freezing and thawing was repeated twice. Finally, an aliquot (100 µl) of the supernatant was added to 2.9 ml of potassium phosphate buffer (50 mM, pH 6.0) containing O-dianisidine dihydrochloride (0.167 mg/ml) and hydrogen peroxide (0.0005%). A Beckman DU-64 spectrophotometer was used to measure the absorbance at 460 nm over 2 minutes. One unit of MPO activity was set at that which degraded 1 µmole of H_2O_2 per minute at 25°C. Circulating leukocyte counts were performed from blood drawn by cardiac puncture. Blood (20 µl) was mixed with 180 µl of 3% acetic acid. White blood cells were counted using a Neubauer chamber under light microscopy.

Tissue was fixed in Bouin's solution and embedded in paraffin for staining either with Masson's trichrome stain for collagen or alternatively, with hematoxylin and eosin for routine histology. Immunohistochemical detection of iNOS was done in frozen tissue sections (5-10 µM) fixed with 1% formaldehyde. Vectastain Elite ABC kits from Vector Labs (Burlingame, CA) were used, which employ a diaminobenzidine (DAB) substrate for identification and hematoxylin as a background stain. Nitrotyrosine immunoreactivity was alone in paraformaldehyde fixed tissues using DAB peroxidase as the visualizing agent with antinitrotyrosine antibodies (Example 1). A peroxidase block was used to inhibit the false positive signal given by endogenous peroxidases. Tissue thickness was determined from the ratio of weight to length (mg/cm). Ileal water content was determined from the wet to dry weight ratios.

All data are expressed as the mean ± standard error (Table 4). Groups were compared by analysis of variance and, when necessary, a Bonferroni-adjusted t-test was used to determine differences between individual groups. The software package Instat was used to determine if tests were appropriate.

Example 8: Detection of inducible nitric oxide synthase RNA expression

RNA was extracted from guinea pig ileum by the guanidine thiocyanate extraction method immediately after collection. First strand cDNA's were synthesized using the reverse primer MHI.3R for inducible nitric oxide synthase (iNOS) (Reverse transcription system, Promega, Madison, WI). First strand cDNA templates were amplified by the polymerase chain reaction using the 22 base primers MHI.3F and MHI.3R for iNOS (Taq-DNA polymerase, Promega, Madison, WI). Denaturation, annealing and elongation temperatures were 95, 60 and 72 °C, respectively, for 1 minute each for 30 cycles. PCR products were separated in a 2% agarose gel and visualized by ethidiuim bromide staining.

Oligonucleotide primers were based on the sequence of a conserved region of mouse and human iNOS. The sense primers (MHI.3F) were as follows: 5'-TCGAAACAACAGGAACCTACCA-3' and 5'-ACCATCCAAGGACAACAAAGAT-3' and the antisense primers used were (MHI.3R) 5'-ACRGGGTGATGCTCCAGACA-3' and 5'-ACAGGCCTCGTAGTGGGGACA-3'.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A monoclonal antibody which binds specifically to nitrotyrosine.

2. A monoclonal antibody which binds specifically to nitrotyrosine and which is detectably labeled with a radioisotope, a fluorophore, a chromophore, a metal atom, a hapten, a chemical crosslinking agent, biotin, avidin, streptavidin or an enzyme.

3. A conjugate comprising a monoclonal antinitrotyrosine antibody linked to a diagnostic agent.

4. The conjugate of Claim 3 in which the diagnostic agent comprises a radioactive label.

5. The conjugate of Claim 3 in which the diagnostic agent comprises an enzyme.

6. A conjugate comprising a monoclonal antinitrotyrosine antibody linked to a therapeutic agent.

7. The conjugate of Claim 6 in which the therapeutic agent comprises an anti-inflammatory agent.

8. The conjugate of Claim 6 in which the therapeutic agent comprises an anti-tumor agent.

9. A hybridoma which produces monoclonal antibodies which bind specifically to nitrotyrosine.

10. A method for detecting a pathological condition in an animal or in a human in which an abnormal level of nitrotyrosine is associated with the pathological condition, wherein binding of antinitrotyrosine antibodies to

nitrotyrosine is detected in a test sample, comprising the steps of:

a) preparing a test sample of tissue or body fluid from an animal or human for binding of antibodies;

b) contacting a preparation of antinitrotyrosine antibodies with the sample in a manner that allows for the specific binding of the antibodies; and

c) using a method of detection of the antinitrotyrosine antibodies to measure the extent of antinitrotyrosine antibody binding in the test sample.

11. The method of Claim 10 comprising immunohistochemical staining.

12. The method of Claim 10 comprising immunoblotting.

13. The method of Claim 10 comprising enzyme-linked immunosorbent assay (ELISA).

14. The method of Claim 10 comprising radioimmunoassay.

15. The method of Claim 10 comprising immunoprecipitation.

16. The method of Claim 10 in which step c is semiquantitative for the level of nitrotyrosine in the sample.

17. The method of Claim 10 wherein the pathological condition is selected from the group consisting of arthritis, infection, illeitis, colitis, adult respiratory distress syndrome and atherosclerosis.

18. A method for delivering, in a living animal or human, a therapeutic agent to a site of inflammed living tissue having abnormally high levels of nitrotyrosine, wherein an antinitrotyrosine antibody within a therapeutic agent-antinitrotyrosine antibody conjugate binds to nitrotyrosine at the site, comprising the steps of:

a) producing a therapeutic agent-antinitrotyrosine antibody conjugate by linking purified therapeutic agent to purified antinitrotyrosine antibody; and

b) administering an appropriate preparation of the therapeutic agent-antinitrotyrosine antibody conjugate to an animal or human by a route appropriate for the site of tissue having abnormally high levels of nitrotyrosine.

19. The methods of Claim 18 in which the therapeutic compound is selected from the group consisting of antioxidants, steroids, anti-tumor agents and enzymes.

20. A method for delivering, in a living animal or human, a diagnostic agent to a site of tissue having abnormally high levels of nitrotyrosine, wherein an antinitrotyrosine antibody within a diagnostic agent-antinitrotyrosine antibody conjugate binds to nitrotyrosine at the site, comprising the steps of:

a) producing a diagnostic agent-antinitrotyrosine antibody conjugate by linking purified therapeutic agent to purified antinitrotyrosine antibody; and

b) administering an appropriate preparation of the diagnostic agent-antinitrotyrosine antibody conjugate to an animal or human by a route appropriate for the site of tissue.

21. A method for detecting, in a living animal or human, a site of tissue having abnormally high levels of nitrotyrosine, wherein an antinitrotyrosine antibody within a diagnostic agent-antinitrotyrosine antibody conjugate binds to nitrotyrosine at the site, comprising the steps of:

a) producing a diagnostic agent-antinitrotyrosine antibody conjugate by linking purified therapeutic agent to purified antinitrotyrosine antibody;

b) administering an appropriate preparation of the diagnostic agent-antinitrotyrosine antibody conjugate to an animal or human by a route appropriate for the site of tissue suspected of having abnormally high levels of nitrotyrosine; and

c) detecting the diagnostic agent using appropriate means.

22. A method of therapy comprising administering to an animal or to a human a compound linked to an antinitrotyrosine antibody, to reduce symptoms of inflammation.

23. A method of therapy comprising administering to an animal or to a human a compound linked to an antinitrotyrosine antibody, to inhibit the growth of cancerous cells.

24. A method of therapy comprising administering to an animal or to a human antinitrotyrosine antibodies which do not bind complement, to reduce the symptoms of an autoimmune disorder caused by endogenously produced antitrotyrosine antibodies which bind complement.

25. A kit comprising monoclonal antinitrotyrosine antibody and reagents for detecting the monoclonal antinitrotyrosine antibody.

26. A kit of Claim 25 wherein the reagents for detecting the monoclonal antibody include immunofluorescent reagents or immunohistochemical reagents.

FIG. 1

FIG. 2

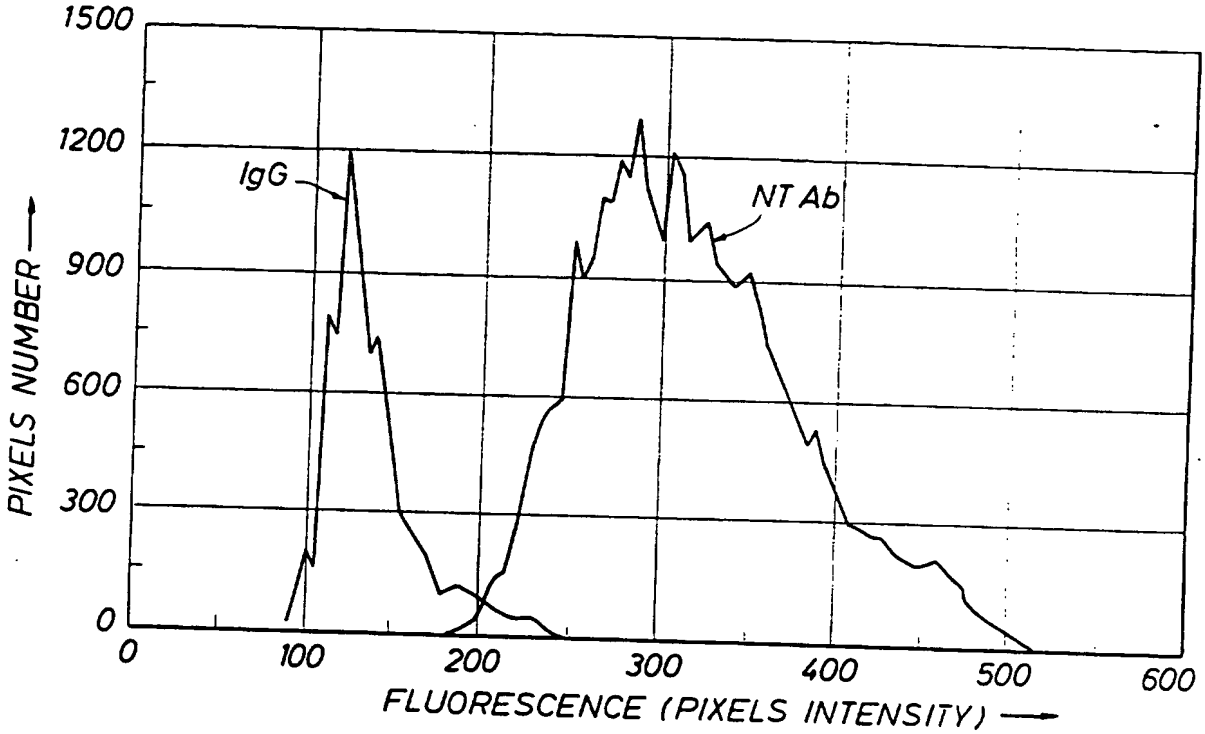

1500
1200
900
600
300
0
PIXELS NUMBER
IgG
NT Ab
0
100
200
300
400
500
600
FLUORESCENCE (PIXELS INTENSITY)

FIG. 3

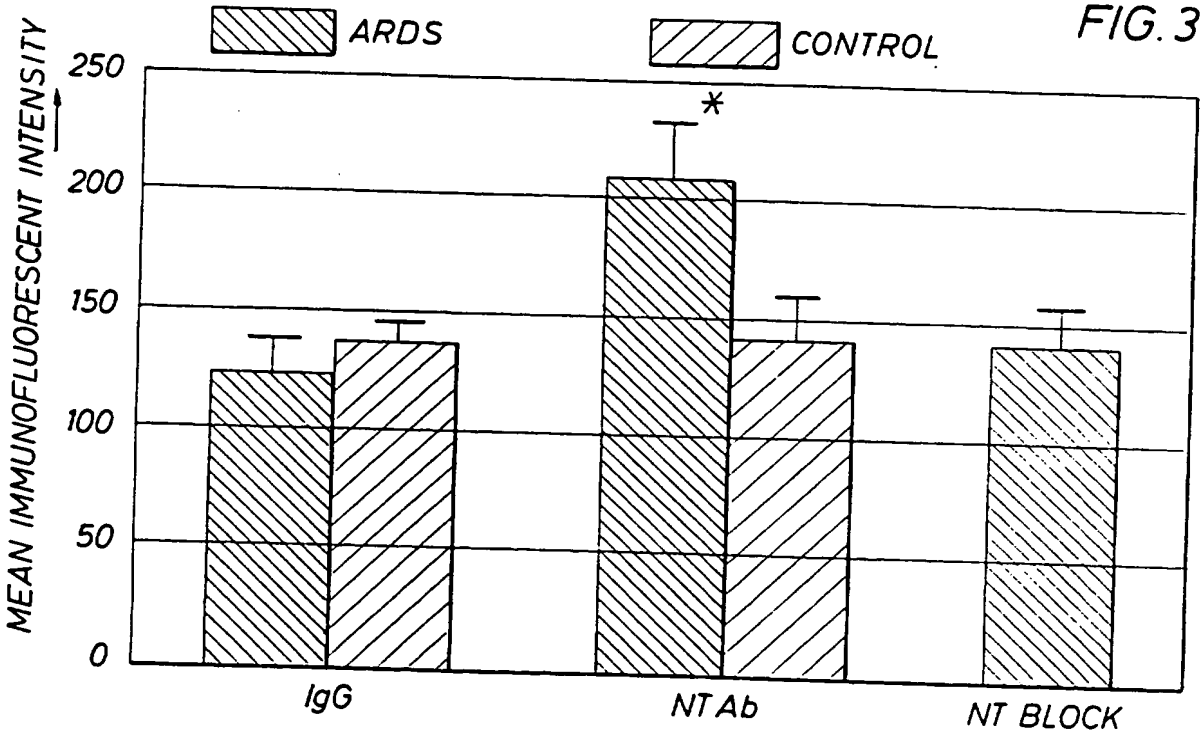

ARDS
CONTROL
MEAN IMMUNOFLUORESCENT INTENSITY
250
200
150
100
50
0
*
IgG
NT Ab
NT BLOCK

FIG. 4

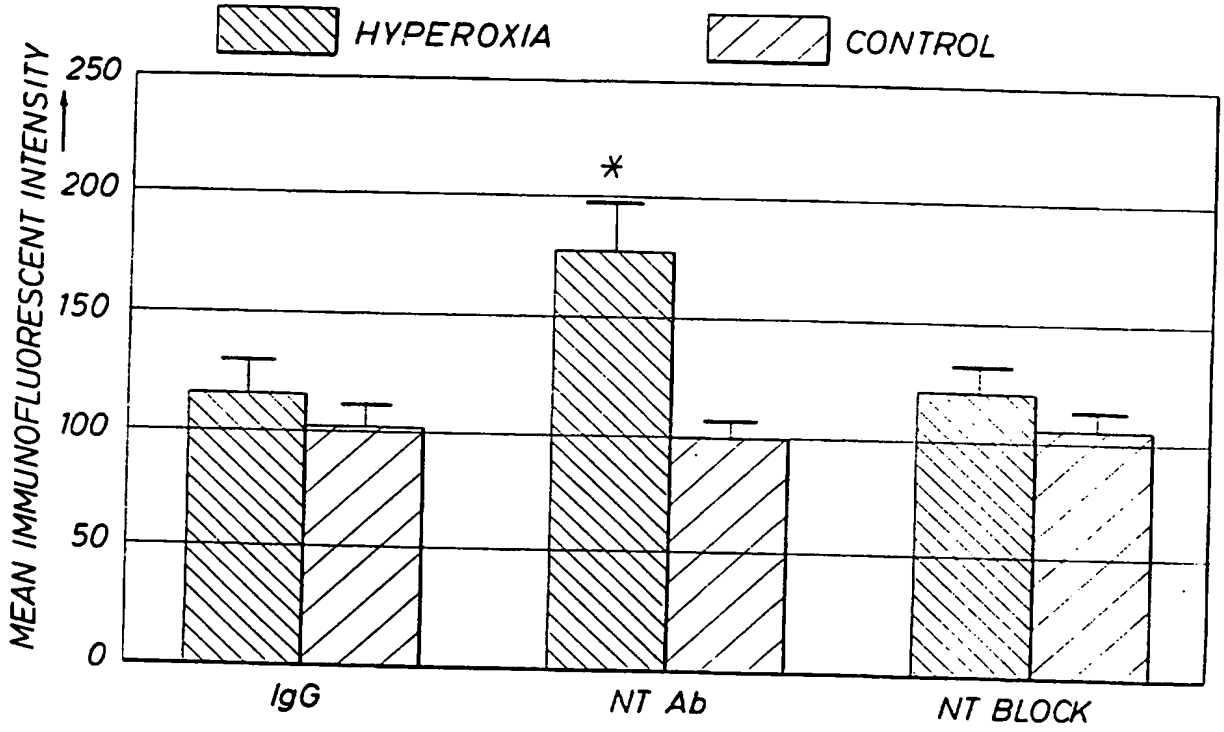
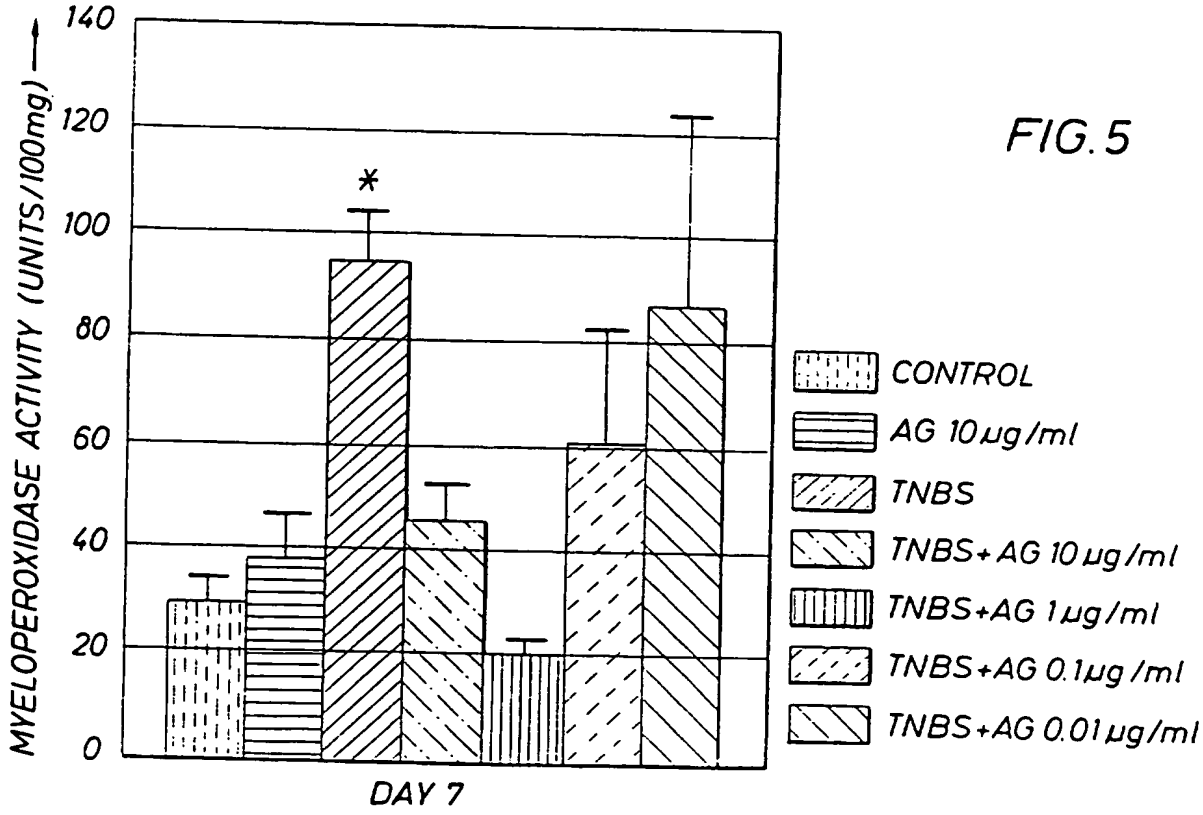

HYPEROXIA
CONTROL
MEAN IMMUNOFLUORESCENT INTENSITY
250
200
150
100
50
0
*
IgG
NT Ab
NT BLOCK

FIG. 5


MYELOPEROXIDASE ACTIVITY (UNITS/100mg)
140
120
100
80
60
40
20
0
*
DAY 7
CONTROL
AG 10µg/ml
TNBS
TNBS+AG 10µg/ml
TNBS+AG 1µg/ml
TNBS+AG 0.1µg/ml
TNBS+AG 0.01µg/ml

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09843

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07K 16/18; G01N 33/53, 33/531, 33/532, 33/558, 33/60; C12N 5/12
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/388.1, 388.85, 388.9, 391.3, 391.5, 391.7, 391.9; 435/7.5, 7.9, 7.92, 240.27, 810; 436/504, 515, 516, 804

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US, A, 5,403,834 (MALFROY-CAMINE ET AL) 04 April 1995, col. 2, lines 46-49.	6, 8
X -- Y	Biol. Chem. Hoppe-Seyler, Volume 375, No. 2, issued February 1994, J. S. Beckmann et al, "Extensive Nitration of Protein Tyrosines in Human Atherosclerosis Detected by Immunohistochemistry", pages 81-88, especially pages 81 and 88.	1, 9 ---------- 2-8, 10-17, 25-26
Y	Clin. Chem., Volume 27, No. 11, issued 1981, E. D. Sevier et al, "Monoclonal Antibodies in Clinical Immunology", pages 1797-1806, especially pages 1800-1802.	1-17, 25-26
Y	Nature, Volume 289, issued January 1981, T. Davies, "Magic Bullets", pages 12-13, see pages 12-13.	6-8

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
27 SEPTEMBER 1995

Date of mailing of the international search report
03 NOV 1995

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09843

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biochem Journal, Volume 125, issued 1971, M. Helman et al, "Isolation of Nitrotyrosine-containing Peptides by Using an Insoluble-Antibody Column", pages 971-974, especially page 971.	1-17, 25-26
Y	Nature, Volume 256, issued 07 August 1975, G. Kohler et al, "Continuous Cultures of Fused Cells Secreting Antibody of predefined Specificity", pages 495-497, see the entire document.	1-17, 25-26
Y	Archives of Biochemistry and Biophysics, Volume 298, No. 2, issued 01 November 1992, H. Ischiropoulos et al, "Peroxynitrite-Mediated Tyrosine Nitration Catalyzed by Superoxide Dismutase", pages 431-437, especially page 431.	1-17, 25-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09843

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-17, 25-26

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09843

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/388.1, 388.85, 388.9, 391.3, 391.5, 391.7, 391.9; 435/7.5, 7.9, 7.92, 240.27, 810; 436/504, 515, 516, 804

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-17 and 25-26, drawn to a monoclonal antibody, a conjugate, a hybridoma, an in vitro method for detecting a pathological condition and a kit, classified in Classes 530 and 435, Subclasses (388.9 and 391.3+) and (240.27, 7.21+ and 810), respectively.
II. Claims 18-19 and 22-23, drawn to a method for delivering in a living animal or human a therapeutic agent, classified in Class 424, Subclass 178.1+.
III. Claims 20-21, drawn to a method for delivering in a living animal or human a diagnostic agent, classified in Class 424, Subclass 9.1+.
IV. Claim 24, drawn to a method of therapy, classified in Class 424, Subclass 175.1.
Groups I-IV do not share the same or a corresponding special technical feature, because they represent different inventive endeavors. The monoclonal antibody, conjugate, hybridoma, kit and the in vitro method for detecting a pathological condition in Group I would not suggest the methods in Groups II-IV. The method in Group II would not suggest the methods in Groups III and IV. The method in Group III would not suggest the method in Group IV. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.